THE MOLECULAR WEIGHT OF ANTIBODIES*

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It is now generally accepted that antibodies are actually modified serum proteins. The work of Felton on the concentration and nature of pneumococcus antibodies (1) and a series of quantitative analytical studies on the precipitin reaction and its mechanism (2) have contributed to the adoption of this conclusion, and have recently led to the isolation of serum protein fractions of which up to 98 per cent of the protein present could be accounted for as antibody by actual chemical analysis (3). The availability of material of such high antibody content suggested the present study.

The determination of the molecular weight of antibodies is of interest in a number of connections. Knowledge of this constant would be expected to throw light on the relation of antibodies to normal serum proteins and on the mechanism of antibody formation. Such a study should also permit chemical formulas to be written for some of the limiting compounds formed in antigen-antibody combination, a subject which will be taken up in another communication. Finally, should differences be found in antibodies produced by different species of animals, this would not only be of physiological interest but might also be of importance for serum therapy. Preliminary studies on antibody particle size have already been reported (2 *d*,[1]

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[1] Heidelberger and Kendall (2 *d*), page 570.

and 4–7) and in the present communication details of our experiments (7) are given.

In this study sedimentation constants were determined in the Svedberg ultracentrifuge (8), in which centrifugal force at high rotational speeds overcomes diffusion and permits the evaluation of particle size from the rate of sedimentation. The material used included whole normal sera and antisera, normal globulin, immune globulin containing up to 50 per cent of anti-egg albumin, Type I pneumococcus antibody concentrate prepared from horse antisera by the Felton method (1), and pneumococcus anticarbohydrate containing up to 98 per cent of antibody, prepared as in Reference 3 from horse and rabbit antisera.

EXPERIMENTAL

Methods

In all but one instance (Experiment 7) the Lamm scale method (9), modified as described by McFarlane (10) and one of us (11), was used for recording the course of sedimentation in the ultracentrifuge. The projection system used was that described previously (11).[2] With this method an equidistant scale is photographed at intervals through the rotating cell. These photographs are compared microscopically with those from a reference scale (11) obtained from a run under similar conditions but with the cell filled with the buffer or salt solution used in the actual run. The displacements of the scale lines, Z, from their positions on the reference scale are plotted as ordinates against the corresponding positions in the cell as abscissae (distance from the axis of rotation) yielding a curve like that in Fig. 1, which shows the sedimentation diagram for a mixture of serum albumin and lactoglobulin. If the resolving power of the centrifuge is sufficient and if the sedimentation constants are not too similar, each particle size will be represented as a peak on the sedimentation diagram as in Fig. 1, in which the heavy line gives the experimental values, and the broken lines indicate in part the two individual curves which give rise to the heavy curve. Since

$$Z = k \cdot \frac{dc}{dx}$$

where k depends on the refractive increment of the sedimenting substance and on known apparatus constants, it is evident that integration of the entire individual curve corresponding to a single peak will give the concentration, c, of the substance having the sedimentation constant of that peak. From the sedimentation

[2] Pederson (11), page 48.

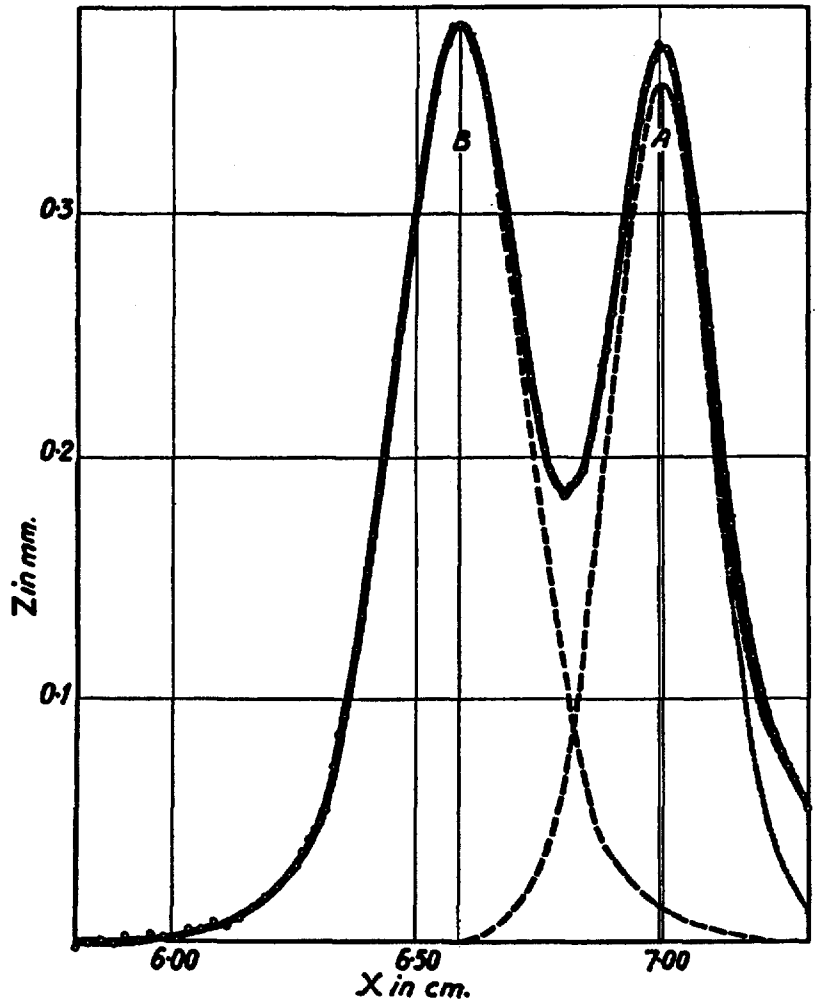

FIG. 1. Analysis of mixture of serum albumin (A) and lactoglobulin (B) in the ultracentrifuge. The experimental curve (solid line) was analyzed to give the individual curves for each component (broken lines). The sedimentation diagrams have been analyzed similarly in the present study.

diagram obtained by the Lamm method one thus obtains both the sedimentation constants (from the change in position of the peaks with time) and the concentrations of the molecules in the solution under investigation. The following

values were used for the refractive index increment, α, at wave length $\lambda = 436$ mμ: for egg albumin, $\alpha = 0.001885$; for serum albumin, $\alpha = 0.001947$; for serum globulin, $\alpha = 0.001967$ (12).

All runs were made at 51,000 and 59,000 R.P.M., corresponding to 190,000 and 250,000 times gravity, respectively. The higher speed was used for most of the rabbit material.

All sedimentation constants were corrected for density and viscosity in the usual way, and in addition for the viscosity due to the protein itself (von Mutzenbecher (13)), since otherwise the sedimentation constants for the faster sedimenting molecules are not comparable.

Except for Experiment 7, all sera and antibody solutions were dialyzed in cellophane against 0.2 M sodium chloride solution, under pressure when concentration of the solution was necessary. Nitrogen analyses were run on all solutions by the micro Kjeldahl method. Values for milligrams of total N per milliliter were transformed to grams of protein per 100 ml. of solution by multiplication by 0.632 for the serum proteins and 0.645 for egg albumin. The precipitin content of all antisera and antibody solutions was determined by the absolute methods given in References 3 and 2*b*.

Rabbit serum globulin was prepared for comparison with whole rabbit sera by dilution of 5 ml. of serum with an equal volume of water and addition of 9 ml. of sodium sulfate solution saturated at 37°C. After centrifugation the precipitate was taken up in 10 ml. of warm sodium sulfate solution at the final concentration and again centrifuged. The globulin was finally taken up in 5 ml. of water and run through a small Chamberland L2 filter.

The Felton solutions (1) were prepared by pouring horse serum into 20 volumes of chilled 0.01 M phosphate buffer at pH 5.5 (calculated), collecting the precipitate, and redissolving it in 0.9 per cent sodium chloride solution.

The dissociated antibody solutions were prepared by dissociation of the washed specific precipitates with strong salt solution (3), and in one instance with barium hydroxide and barium chloride (3).

The analyses and the experimental results from the centrifuge runs are summarized in the tables and Figs. 2 and 3. The latter show the relative distribution of the amount of substance used with respect to particles of the sedimentation constants determined from the sedimentation diagrams. The area of the rectangles is proportional to the relative concentration of the molecules having the given sedimentation constant. These rectangles also indicate whether the corresponding peak in the sedimentation diagram is almost homogeneous or not, since their breadth is put equal to a sedimentation constant unit ($1 \cdot 10^{-13}$) for the homogeneous peaks, whereas the non-homogeneous peak is indicated by a broader rectangle. The letters *c* and *s* give, respectively, the concentration in gm. per 100 ml. of solution and the sedimentation constant times 10^{13} for the given component. In Figs. 2 and 3 the rectangles corresponding to egg albumin, serum

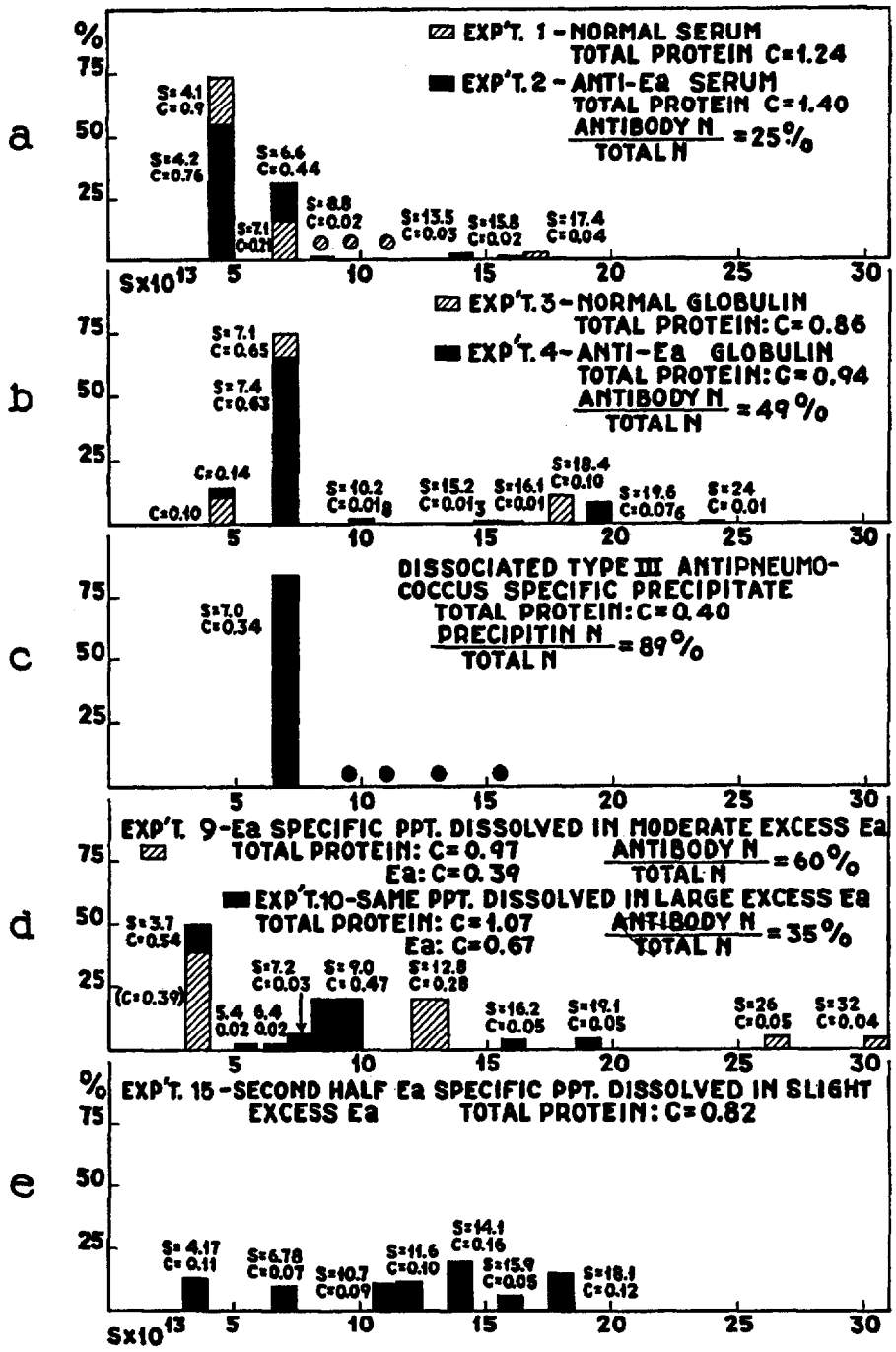

FIG. 2. Quantitative diagrammatic representation of molecular species found in ultracentrifuge runs on rabbit sera and antibody solutions. Explanation at bottom of page 396.

albumin, and serum globulin (Ea, Sa, and Sg[3]) are always placed at the normal s value for these substances, whereas for all the other substances studied the rectangles are placed at the experimentally determined s value. The error in the determination of the absolute s value for the faster sedimenting molecules is generally $\pm$ 5 per cent and may occasionally increase to $\pm$ 10 per cent in the most complicated diagrams. For the homogeneous solutions the errors in s are smaller. Circles in the diagrams indicate inhomogeneous material.

Observations on Rabbit Sera and Antibody Solutions

Experiment 1. Sterile Normal Rabbit Serum 4.36$_0$ Containing No Added Preservative.—This serum contained 10.4 mg. total N per ml. After an arbitrary deduction of 0.3 mg. for non-protein N the serum contained 7.2 mg. albumin and 2.9 mg. globulin per ml. by the Howe micro method (14). Thus 69 per cent of the total N was due to albumin and 28 per cent to globulin. The concentrations calculated from the sedimentation diagram were 73 per cent[4] of albumin, in good agreement with the analytical value, while the globulin component was only 17 per cent of the total, or considerably less than the analytical value. However, the sedimentation diagram also showed the presence of several small peaks which partially explain the low value for the normal (principal) globulin peak. It has been found by one of us[5] and by von Mutzenbecher (13) that normal globulin always contains small amounts of molecules with s about $18 \cdot 10^{-13}$. This serum, too, showed the presence of molecules with $s = 17.4 \cdot 10^{-13}$ (3 per cent). The ratio of the concentration of the principal globulin component to that of the 17 component was 5.7. Further details of the run are to be found in Fig. 2 *a*.

Experiment 2. Sterile Rabbit Anti-Egg Albumin Serum 4.36$_2$ Containing No Added Preservative.—This serum was obtained from the same rabbit as the normal serum, after two courses of intravenous injections, using 5 mg. of alum-precipitated Ea per injection (*cf.* 2 *e*). Of the 11.7 mg. of protein N per ml. of the serum, 6.1 mg. were globulin N and 5.6 mg. albumin N (Howe method). The serum contained 3.1 mg. of anti-Ea N per ml., or 26 per cent of the protein N. The sedimentation diagram also showed a strong increase over the normal serum in the globulin peak and a corresponding decrease in the other peaks. The analysis gave 48 per cent for albumin, while the sedimentation diagram gave 54 per cent. For the globulin the values were 52 and 31 per cent, respectively, in poor agreement. The ratio between the amounts of the 7 component and the 17 component was 22; that is, the increase in the principal globulin component was greater than in the smaller, heavier one (see Fig. 2 *a*).

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[3] These designations are also used for these proteins throughout the experimental part.

[4] All subsequent values are given as per cent of the total protein calculated from the amount of total N present.

[5] Pedersen, K. O., unpublished results.

Experiment 3. Normal Rabbit Globulin 4.36$_0$.—The solution was obtained from the serum used in Experiment 1 as previously described. The sedimentation diagram showed 76 per cent of the principal globulin, 11 per cent of albumin, and, in addition, 12 per cent of a component with $s_{20} = 18 \cdot 10^{-13}$ and other heavy components in low concentration. The ratio of the normal globulin to the 18 component was 6.5 (Fig. 2*b*).

Experiment 4. Immune Rabbit Globulin 4.36$_2$.—This was obtained from the anti-Ea serum used in Experiment 2. Although 50 per cent of the protein present was specifically precipitable by Ea the sedimentation curves were scarcely different from those of the corresponding normal globulin. The ratio between the normal and the faster sedimenting globulin was 8.3, a small increase (Fig. 2*b*).

Experiments 5 and 6. Immune Globulin.—These experiments on immune globulin obtained from two other anti-Ea sera, 4.37$_2$ and 4.31$_2$, gave essentially the same result as Experiment 4. The ratio between the two globulin components in both experiments was 12.

Experiment 7. Rabbit Type III Pneumococcus Anticarbohydrate 3.50$_1$.—The water-clear solution was prepared by salt dissociation according to Reference 3 from the serum of rabbit 3.50, which had been injected with formalinized Type III pneumococcus. The method yields only a portion of the total antibody. The solution was preserved with 0.01 per cent of merthiolate. Although 90 per cent of its protein was precipitable by Type III pneumococcus specific polysaccharide it showed $s = 7.0 \cdot 10^{-13}$, the value characteristic as well for the principal component of normal rabbit globulin. The light absorption method was used for this run.

Experiment 8. Rabbit Type III Pneumococcus Anticarbohydrate 3.51$_1$.—The water-clear solution (3) was prepared from the serum (preserved in the cold with 0.01 per cent of merthiolate) of another rabbit, and 89 per cent of its protein was specifically precipitable by the homologous specific polysaccharide. It was homogeneous (84 per cent of the analytical value)[6] in the ultracentrifuge, showing only the component $s_{20} = 7.0 \cdot 10^{-13}$. The few minor peaks present were so small that no sedimentation constants could be calculated (Fig. 2*c*).

The following experiments were carried out in attempts to gain information regarding the size of the molecules in the inhibition zone of the precipitin reaction, in which specific precipitation is inhibited by an excess of antigen.

Experiments 9 and 10. Egg Albumin-Anti-Egg Albumin Specific Precipitate Dissolved in Excess Egg Albumin Solution.—8.5 ml. of serum 4.36$_2$, preserved with 0.01 per cent of merthiolate, were divided into two equal portions, diluted with saline, and precipitated with a total of 1.73 mg. of Ea N, leaving a slight excess

[6] Five determinations from the sedimentation diagrams agreed within 4.5 per cent.

of antibody to insure the presence of all added Ea in the precipitate (2*e*). After three washings with 10, 5, and 5 ml. of chilled saline the specific precipitates were recombined, suspended in 3.0 ml. of 0.2 M NaCl solution, and treated with 0.5 ml. portions of Ea solution (2.59 mg. Ea N per ml.) at 5 to 10 minute intervals, until only traces of insoluble material remained, clearing taking place when 4.0 ml. of Ea solution had been added. It was found best to warm the mixture at 35–38°C. in the presence of a drop of toluene, as solution of the precipitate required a number of hours at 0°. The solution was transferred quantitatively to a cellophane tube, dialyzed against several changes of 0.2 M NaCl solution, and made up to 10.0 ml. with 0.2 M NaCl solution. For Experiment 9, part of the solution was diluted 1:1 with 0.2 M NaCl solution, for Experiment 10, 2.0 ml. were mixed with 0.50 ml. of Ea solution containing 4.32 mg. Ea N, again dialyzed against the salt solution, and diluted 1:1 as before. Since the amount of Ea N in all samples was known, total N − Ea N = antibody N present.

The sedimentation diagram from Experiment 9 (Fig. 2*d*) showed as the main peak (besides that due to Ea) a rather inhomogeneous one with a mean s of about $13 \cdot 10^{-13}$ (29 per cent of the analytical protein value). There were also several other peaks with higher values, the two largest showing $s = 26 \cdot 10^{-13}$ (5 per cent) and $32 \cdot 10^{-13}$ (4 per cent). Experiment 10, in which a large excess of Ea was added, gave a quite different sedimentation diagram (Fig. 2*d*). The main peak was even less homogeneous and showed a lower mean s, about $9 \cdot 10^{-13}$ (44 per cent). The peak was very broad and of a peculiar shape with a sharp limit at the slower sedimenting side. This sharp boundary sedimented at the rate of normal globulin ($s_{20} = 7.2 \cdot 10^{-13}$). Most of the faster sedimenting peaks had disappeared from the diagram. This change may be explained by recent studies (15) which indicate that a lighter component in relatively high concentration causes a shift toward lower sedimentation constants for the heavier components present, a change considered as a dissociation of the heavier components.

Experiments 11, 12, 13, 14, and 15.—These experiments represent similar runs, except that in Experiments 11, 13, and 14 approximately one-half of the antibody in a portion of serum 4.36_2 was precipitated with Ea and redissolved in different amounts of Ea, while the remainder of the antibody in the serum was precipitated for Experiments 12 and 15 and dissolved in excess antigen. The experiments confirmed the previous ones in so far as a low s (but greater than that of globulin alone) was found in the experiments with high Ea concentrations, and high s values were found with low Ea concentrations. The influence of the Ea is best seen from Table III.

The behavior of the antibody was noteworthy in Experiment 15, in which the Ea content was kept at the minimum. The sedimentation diagram (Fig. 2*e*) showed a number of sharp, well defined peaks which appear due to molecules of very definite size, possibly formed by the combination of antigen and antibody in simple proportions. If further work should show this to occur with regularity it would be necessary to assume that a larger excess of Ea dissociates the original soluble complexes of large size, converting them into compounds of lower molecular

weight. There would thus be an accord between Pedersen's explanation (15), the ideas expressed in References 2 and 16, and Marrack's views (17).

Experiment 13 was a repetition of Experiment 11 on another portion of the solution 3 days later to establish whether or not the s value, higher than that of antibody globulin itself, was due to a relatively slow disaggregation of the precipitate. The value obtained, however, was not lower.

Observations on Horse Sera and Antibody Solutions

Experiment 16. Felton Solution from Normal Horse Serum.—The slight precipitate formed on pouring normal horse serum into 20 volumes of chilled 0.01 M phosphate buffer at pH 5.5 (1) was redissolved in 0.9 per cent NaCl solution and let stand in the cold for several days with a little toluene. A portion of the clear supernatant was used for the centrifuge run after dialysis against 0.2 M NaCl solution. As opposed to the Felton solutions from antipneumococcus sera (see below), very little of the component $s = 18 \cdot 10^{-13}$ was present, the main component (60 per cent, not homogeneous) showing $s_{20} = 8.1 \cdot 10^{-13}$ (Fig. 3 *a*).

Experiment 17. Felton Solution from Type I Antipneumococcus Horse Serum.—This was prepared as in Experiment 16 from a sample of sterile, unpreserved serum.[7] The serum contained 0.64 mg. of Type I anticarbohydrate N per ml., the low antibody content being perhaps responsible for the relatively small proportion (29 per cent) of antibody in the Felton solution and also (>51 per cent) in the dissociated antibody used in the next experiment. Before use a portion of the anti-C was removed from the serum by precipitation with pneumococcus C substance (18). Besides several small peaks the sedimentation diagram showed two main components, one with $s_{20} = 6.8 \cdot 10^{-13}$ (40 per cent) and another with $s_{20} = 18.3 \cdot 10^{-13}$ (42 per cent) (Fig. 3 *a*).

Experiment 18. Dissociated Type I Pneumococcus Anticarbohydrate.—An antibody solution was prepared by salt dissociation of the washed precipitate (3) from the above serum and Type I pneumococcus specific polysaccharide (19) (referred to below as S I). Only a portion of the antibody is recovered by this method. Owing to an accident the analysis for precipitin could be made only on a portion of the solution which had been exposed to deterioration through extensive manipulation, the value obtained being 51 per cent of the total N. The dissolved protein was almost homogeneous in the ultracentrifuge, showing $s_{20} = 18.5 \cdot 10^{-13}$ (64 per cent of the analytical value).

Experiment 19. Dissociated Type I Pneumococcus Anticarbohydrate.—The solution used in this experiment was obtained similarly from a later bleeding of the same horse, the serum containing only 0.43 mg. of anticarbohydrate N per ml. The precipitin N content of the solution was 61 per cent of the total and the sedimentation diagram showed practically only a single component of $s_{20} = 18.2 \cdot 10^{-13}$ (93 per cent of the total protein (analytical)) (Fig. 3 *b*).

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[7] This serum was kindly supplied by Dr. S. Gard of the Statens bakteriologiska Laboratorium, Stockholm, to whom we wish to express our thanks.

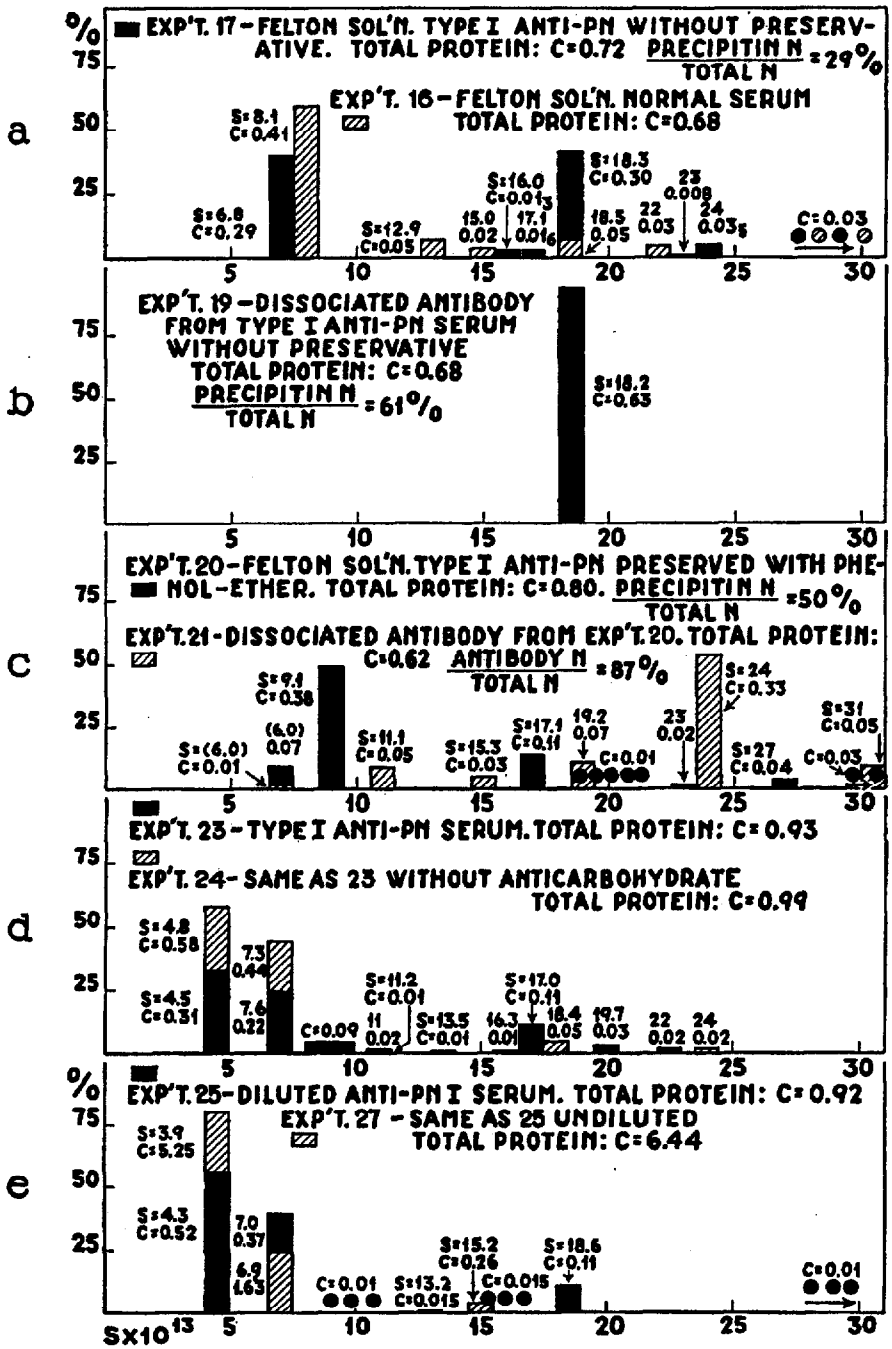

FIG. 3. Quantitative diagrammatic representation of molecular species found in ultracentrifuge runs on horse sera and antibody solutions. Explanation at bottom of page 396.

Experiment 20. Felton Solution from Type I Antipneumococcus Serum Preserved with Phenol and Ether.—The serum, No. 610,[8] contained 1.50 mg. of Type I anticarbohydrate per ml. Before use it was absorbed with C substance and pneumococcus protein. The Felton solution was prepared as in the previous instances and 50 per cent of its protein content was precipitable by S I. In the sedimentation diagram the main component (48 per cent) was very inhomogeneous and had a low s, namely $9.1 \cdot 10^{-13}$. Several other faster sedimenting components were present, the largest having $s_{20} = 17.1 \cdot 10^{-13}$ (14 per cent), but even these were not as homogeneous as in the preceding Felton solution, possibly owing to the exposure of the serum proteins to phenol and ether during storage. This would be in accordance with McFarlane's findings (20) with alcohol-ether-treated serum.

As soon as the 17 component had reached the bottom of the cell the centrifuge was stopped and the upper portion of the cell contents was pipetted off. This contained a small amount of heat-coagulable protein but gave only a faint opalescence with a 1:20,000 solution of S I. The bottom layer in the cell, however, gave heavy specific precipitation. The antibody function thus occurs with the heavier components.

Experiment 21. Dissociated Type I Pneumococcus Anticarbohydrate from Felton Solution of Experiment 20.—Although 87 per cent of the protein in this preparation (79 A_1, Reference 3) was specifically precipitable, the material was polydisperse, again possibly owing to the long exposure of the serum proteins to phenol and ether. The s of the main component (53 per cent) had in this case increased to $23.9 \cdot 10^{-13}$ but several other components were present in concentrations ranging from 2 to 11 per cent of the analytical value. The increase in the proportion of protein with higher sedimentation constants with increase in antibody content is readily seen in the comparison of Experiments 20 and 21 in Fig. 3*c*.

Experiment 22. Type I Pneumococcus Anticarbohydrate Dissociated by Barium Hydroxide.—The specific precipitate used for dissociation (solution 79 E, Reference 3) was obtained in part from the Felton solution used for Experiments 20 and 21, but in spite of this the sedimentation diagram was quite different from that of Experiment 21, all diagrams at comparable times showing displacement toward smaller s values. The two main components were $s_{20} = 18.7 \cdot 10^{-13}$ (46 per cent) and $s_{20} = 23.2 \cdot 10^{-13}$ (26 per cent), while a component of $s_{20} = 9.1 \cdot 10^{-13}$ was responsible for 17 per cent (together with a $13 \cdot 10^{-13}$ component). It is probable that even the brief treatment with barium hydroxide in the cold (3) caused appreciable dissociation of the antibody protein. In accord with this and with the precipitability of 96 per cent of the protein by S I, both the top and bottom portions of the cell contents reacted with S I, the bottom part yielding the heavier precipitate. The centrifuge was stopped just after the 18 component had reached the bottom of the cell.

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[8] This serum was obtained through the courtesy of Dr. William H. Park of the New York City Department of Health Laboratories.

Experiment 23. Type I Antipneumococcus Horse Serum.—This experiment was run on a highly potent Type I antipneumococcus serum,[9] assaying 2.8 mg. of Type I anticarbohydrate N and over 1000 mouse protective units per ml. After dialysis against 0.2 M NaCl solution the serum was diluted to about 1 per cent of protein with the salt solution. The sedimentation diagram showed the normal peaks due to albumin (33 per cent) and globulin (24 per cent) and several rather indistinct peaks, the main one having $s_{20} = 17 \cdot 10^{-13}$ (12 per cent). Between the normal globulin peak and a small peak with $s_{20} = 11 \cdot 10^{-13}$ (2 per cent) the diagram indicated about 10 per cent of inhomogeneous material (Fig. 3*d*).

Experiment 24. Type I Antipneumococcus Horse Serum Deprived of Anticarbohydrate.—2.0 ml. of the above serum were precipitated in the cold with 2.4 ml. of a 1:2000 solution of S I, removing practically all anticarbohydrate. After standing overnight the mixture was centrifuged in a cold room. This serum also was run at a concentration of about 1 per cent protein. The sedimentation diagram showed a considerable reduction in the relative concentration of the faster sedimenting molecules (Fig. 3*d*).

Experiments 25 and 26. Type I Antipneumococcus Horse Serum without Preservative, and the Same Deprived of Anticarbohydrate.—This serum was obtained from a later bleeding of the Stockholm horse and the antibody (0.65 mg. N per ml.) was removed from one portion with S I as described above. Solutions containing about 1 per cent of protein were used. The sedimentation diagrams of even this weak serum indicated a slight decrease in the concentration of the heavier molecules after removal of the antibody. There was also possibly a slight decrease in the albumin peak.

The above experiments were, in general, carried out with a protein concentration of about 1 per cent because of lack of time for runs at several dilutions. This concentration was chosen because it could be expected (15) that changes in the serum would be more marked in dilute solution than in the concentrated serum, in which the effect described in References 10 and 15 might entirely mask the changes. Several experiments were run, however, in which the serum was not diluted, but merely dialyzed against 0.2 M NaCl solution.

Experiments 27 and 28. Undiluted Type I Antipneumococcus Horse Serum, without Preservative, and the Same Deprived of Anticarbohydrate.—The serum was the same as used in Experiments 25 and 26, but undiluted. The sedimentation

[9] The serum was supplied by Dr. Percival Hartley of The National Institute for Medical Research, Hampstead, London, to whom we are also most grateful for laboratory facilities and his personal aid.

diagrams were almost identical, regardless of the presence or absence of antibody, and gave the same values for the concentration of the three distinct peaks present, whereas the same sera showed distinct differences in dilute solution. Most of the faster sedimenting molecules had disappeared from the diagrams of the concentrated sera, the globulin peak had greatly diminished, and that due to the albumin had increased markedly (*cf.* 10) (see Fig. 3*e*, in which a comparison is given of the diluted and undiluted antiserum).

Experiment 29. Same Serum as in Experiment 23, but Undiluted.—The result was very much as above—an increase in the apparent albumin concentration and a decrease in that of the faster sedimenting molecules. It has, however, been amply shown that the amount of antibody is the same at all dilutions (2*d*).

DISCUSSION

In the present study a comparison has been made in the ultracentrifuge of normal and anti-egg albumin sera of known protein and antibody content from the same rabbit, normal and immune rabbit globulin, Felton solutions from normal and Type I antipneumococcus horse sera, and horse and rabbit pneumococcus anticarbohydrate of which up to 98 per cent of the protein present could be accounted for as antibody.

Let us first consider the rabbit material. It is evident from the data summarized in Table I and Fig. 2 that in moderately dilute solution the same sedimentation constant is obtained for the principal globulin component of normal rabbit serum, of isolated normal globulin, of anti-egg albumin serum, and of the corresponding isolated immune globulin. In both of the last up to about 50 per cent of the globulin present was specifically precipitable by egg albumin. Moreover, specimens of Type III pneumococcus anticarbohydrate (Experiments 7 and 8) produced in the rabbit, in which approximately 90 to 95 per cent of the protein present was accounted for as antibody, not only showed the same sedimentation constant but were monodisperse (Fig. 2 *c*), indicating that the constant observed was actually that of the antibody. For all of this material the sedimentation constants varied from 6.6 to $7.4 \cdot 10^{-13}$, with an average of $7.1 \cdot 10^{-13}$, a value close to that found for the principal globulin fraction of other normal mammalian sera studied in Upsala (13, 10, 20, and unpublished data). It appears, therefore, that the molecular weight, at least of the two classes of rabbit antibodies included in this study, is

very close to that of normal serum globulin, about 150,000. The general applicability of this result for rabbit antibodies is further indicated by the finding of the same sedimentation constant, $7 \cdot 10^{-13}$, by Biscoe, Herčik, and Wyckoff (5) for a preparation containing anti-azoprotein, although the proportion of antibody present was not known. Goodner, Horsfall, and Bauer (6), however, reported much antibody in large aggregates in ultrafiltration studies on Type I antipneumococcus rabbit serum.

TABLE I

Observations on Normal and Immune Rabbit Sera, Globulin, and Antibody Solutions

Experiment No.	Material used	Concentration		$s_{20} \cdot 10^{13}$ principal globulin component	$s_{20} \cdot 10^{13}$ globulin component next in amount	Dispersity
		Total N	Antibody N			
		mg. per ml.	mg. per ml.			
1	Sterile, normal rabbit serum, no preservative	1.96		7.1	17.4	Globulin nearly monodisperse
2	Anti-Ea serum from same rabbit, no preservative	2.21	0.6	6.6	13.5	" "
3	Normal globulin, same rabbit	1.36		7.1	18.4	" "
4	Immune (anti-Ea) globulin, same rabbit	1.49	0.73	7.4	19.6	" "
5	Anti-Ea globulin, rabbit 4.37_2	2.90	1.05	7.4	20.7	" "
6	Anti-Ea globulin, rabbit 4.31_2	1.42	0.48	7.3	20.3	" "
7	Rabbit Type III pneumococcus anticarbohydrate 3.50_1	0.11	0.10*	7.0		
8	Same, rabbit 3.51_1	0.64	0.57	7.0		Homogeneous

* Analysis made 2 months previously.

On the basis of the observed sedimentation constant it would appear that antibody in the rabbit is formed either from the principal globulin component of the serum or possibly by the cells or tissues responsible for the building up of the principal component of normal serum globulin. Thus the finding of a molecular weight characteristic of normal globulin might be taken as evidence in favor of the theory

of antibody formation put forward by Breinl and Haurowitz (21) and by Mudd (22), a theory, which, although widely accepted, has up to the present lacked an experimental basis.

TABLE II

Observations on Horse Sera, Concentrates, and Antibody Solutions

Experiment No.	Material used	Concentration		$s_{20} \cdot 10^{13}$ principal component	$s_{20} \cdot 10^{13}$ additional components in largest amounts	Dispersity
		Total N	Precipitin N			
		mg. per ml.	mg. per ml.			
	Normal horse globulin (13)			7.1	10.5, 18.8	Polydisperse
16	Felton solution, normal horse serum	1.07		8.1	12.9, 18.5	"
17	Felton solution, Type I antipneumococcus serum containing no preservative	1.14	0.33	18.3	6.8, 24.2	"
18	Dissociated antibody from same serum	0.92	0.47	18.5		Homogeneous
19	Dissociated antibody from similar serum	1.08	0.66	18.2		"
20	Felton solution, Type I antipneumococcus serum preserved with phenol-ether	1.27	0.63*	9.1	17.1, 27.3	Polydisperse
21	Dissociated antibody from above Felton solution	0.98	0.85*	23.9	19.2, 11.1, 30.6	"
22	Antibody dissociated by $Ba(OH)_2$ method	0.55	0.53*	18.7	9.1, 13.3, 23.2	"
				Globulins		
23	Sterile Type I antipneumococcus serum	1.47	0.33	7.6	17.0, 10.9	"
24	Same serum without anticarbohydrate	1.57		7.3	18.4†	"
25	Sterile Type I antipneumococcus serum containing no preservative	1.46	0.09	7.0	18.6	"
26	Same, without anticarbohydrate	1.70		7.5	20.6	"

* From analyses made 2 to 3 months previously. The solutions had undergone no visible change.

† Experiments 24 and 26 showed smaller amounts of the heavier components than did the whole sera. See Fig. 3 *d*.

As regards antibodies produced by the horse, a more complicated state of affairs is indicated. From the data summarized in Table II and Fig. 3 the following will be noted. The principal component of the Felton solution obtained from fresh, normal horse serum has a sedimentation constant somewhat higher than that of normal serum globulin. On the other hand, there is relatively less material with this sedimentation constant in the Felton solutions prepared from Type I antipneumococcus horse sera and it is accompanied by significant amounts of a component of $s_{20} = 18.1 \cdot 10^{-13}$ and heavier molecules as well. Likewise most of the protein in Type I pneumococcus anticarbohydrate solutions, prepared according to Reference 3 and containing roughly from 50 to 98 per cent of the protein in the form of antibody, showed sedimentation constants of s_{20} = about $18 \cdot 10^{-13}$, except for the material in a salt-dissociated specimen obtained from phenol-ether-treated antiserum, which will be discussed below.

Two preparations of the antibody fraction dissociated by strong salt (3) from the specific precipitate from Type I antipneumococcus horse serum containing no preservative showed homogeneous sedimentation, s being 18.2 and $18.5 \cdot 10^{-13}$. These observations are in accord with conclusions based on diffusion measurements ($2d$),[1] ultrafiltration experiments on a Type I antipneumococcus horse serum by Elford, Grabar, and Fischer (4), and ultracentrifugal studies on Felton concentrates reported in a preliminary note by Biscoe, Herčik, and Wyckoff (5). They do not, however, account for the enormous aggregates postulated by Goodner, Horsfall, and Bauer (6) as a result of the ultrafiltration of Type I antipneumococcus horse serum. All reports indicate, however, that pneumococcus I anticarbohydrate in the horse consists of protein molecules of high molecular weight.

In the horse antibody solutions studied thus far in the work the antibody had been precipitated during its preparation, either with specific polysaccharide or by pouring into water. While the former procedure had not increased the molecular size of rabbit antibody, it seemed possible that precipitation of antibody formed by the horse might result in an aggregation which would not necessarily be entirely reversible, and that this might account for the high sedimentation

constant observed. Two different Type I antipneumococcus horse sera were accordingly diluted and run in the ultracentrifuge before and after removal of the anticarbohydrate. Both showed a notable reduction chiefly in the more rapidly sedimenting molecules, indicating again that the antibody occurred in these fractions of high molecular weight.

Eliminating the values found in Experiments 20 and 21 as out of line, the sedimentation constant of the principal component of the anticarbohydrate-containing solutions varies from 18.2 to $18.7 \cdot 10^{-13}$, with an average of $18.4 \cdot 10^{-13}$. The sedimentation constant alone does not define the molecular weight of the antibody, since sedimentation depends not only on the molecular weight of the particle but also on its shape, and this is at present unknown. By comparison with the molecular weights of molecules having sedimentation constants at about $18.4 \cdot 10^{-13}$, one could guess at a molecular weight of about one-half million, or 3 to 4 times that of normal globulin. It should be noted that a small component of approximately this magnitude is characteristic of the globulin of most normal sera so far investigated, including the rabbit sera shown in Fig. 2. It will be seen from this figure that the amount of this component is not increased in immune rabbit sera or globulin, and, indeed, is not present in appreciable amount in highly purified anticarbohydrate produced by the rabbit. There would thus appear to be a fundamental difference in the mechanism of formation at least of pneumococcus anticarbohydrate in the horse and in the rabbit, in that this antibody arises in the horse by development of an otherwise minor globulin fraction which the rabbit does not use either for the production of pneumococcus anticarbohydrate or for other antibodies (see, however (6)).

Since there are many differences, such as water precipitability, for example, between pneumococcus anticarbohydrate and other antibodies such as diphtheria antitoxin produced by the horse, it is possible that different antigens produce different stimuli in this animal, resulting in a more varied response than in the rabbit. Information along these lines is being sought in this laboratory.

It would be reasonable and tempting to assume that the high molecular weight of pneumococcus anticarbohydrate in the horse is the cause of many of the observed chemical and physiological differences

in this antibody as produced in the horse and in the rabbit. Thus even slight degradation of the specific polysaccharide is sufficient to decrease the amount of rabbit antibody it precipitates (19) without affecting the quantity of homologous horse antibody thrown down, while by more severe degradation it is possible to obtain polysaccharide preparations which do not precipitate rabbit antibody but still throw down that produced by the horse (23). Possibly even the failure of horse anticarbohydrate to sensitize guinea pigs passively to the homologous polysaccharide or to fix complement with it might be ascribed in part to the large size of the horse anticarbohydrate molecule. If these surmises have any basis they would also raise the question as to whether or not the horse is the best animal from which to draw serum for use in antipneumococcus therapy.[10] Experiments have therefore been undertaken along these lines.

It will be observed from Table II and Fig. 3 that the sedimentation behavior of antibody solutions prepared from sera which had been preserved with phenol and ether was more complex than that of antibody solutions obtained from sterile, unpreserved sera. This is in accordance with an earlier finding of McFarlane (20) and is possibly due to the alteration or denaturation of a portion of the serum proteins by the preservative. However, the phenol-ether-treated serum contained more antibody than did the one yielding the simpler solutions and this may also have influenced the results. The use of 0.01 per cent merthiolate[11] did not seem to affect the sedimentation picture.

Brief treatment of the antibody with alkali (Experiment 22) resulted in a shift toward more slowly sedimenting molecules. Even the smallest of these were specifically precipitable, as shown both by analysis of the entire solution and by tests on the upper and lower portions of the cell contents after centrifugation. On the other hand, only the lower portion of the cell contents reacted for antibody in the case of a Felton solution (Experiment 20), providing

[10] Since the completion of this work, this question has been raised by Horsfall, Goodner, and MacLeod (*Science*, 1936, **84**, 579).

[11] The merthiolate used in this investigation was presented by the manufacturers, Eli Lilly and Company, Indianapolis.

additional evidence for the occurrence of the antibody in the more rapidly sedimenting fractions under ordinary conditions.

Antibody solutions of a high degree of purity may thus vary from the monodisperse to the polydisperse in the ultracentrifuge, depending, at least in part, upon the treatment to which the protein has previously been subjected. The property of precipitability by means of the specific polysaccharide is not affected within the range studied and thus extends over considerable differences in molecular size. Nevertheless, for studies on the dispersity of antibodies it would appear desirable to avoid the use of preservatives whenever possible. Furthermore, even in cases in which the presence or absence of antibody is shown by distinct changes in the sedimentation diagrams of diluted sera (Experiments 25 and 26), the diagram produced with the undiluted antiserum may be impossible to distinguish from that obtained with the same serum deprived of its antibody and centrifuged at the same high total protein concentration (Experiments 27 and 28). Thus antibodies, like many other proteins ((15) and page 400), appear to be dissociated into smaller molecules in concentrated protein solutions, and the sedimentation diagram in the undiluted serum becomes more obscure. It therefore seems preferable at present to study the changes caused by immunization in the sedimentation diagrams of dilute sera and antibody solutions, especially since it has been shown that the antibody content of a serum does not depend on the dilution at which the analysis is carried out (2*d*).

Data are also given in Table III and Fig. 2 on the ultracentrifugal behavior of egg albumin-anti-egg albumin precipitates dissolved in an excess of egg albumin. These experiments were made not only because they afforded a means of obtaining solutions in which anti-egg albumin comprised more than 50 per cent of the protein present (Experiments 9, 12, 15), but also because of their theoretical implications. Thus the inhibition zone of the precipitin reaction may be considered due to peptization of the specific precipitate by excess of antigen, or due to the formation of soluble compounds between antibody and relatively much antigen (2*a*, 2*d*, 16, 17). It was hoped that the behavior of the dissolved precipitates in the ultracentrifuge would throw some light on whether the colloid chemical or the classical chemical theory applied.

Actually, interpretation of the results was rendered somewhat difficult, not only by the complex sedimentation diagrams of occasional solutions, but also by the shift toward lower sedimentation constants caused by a larger excess of egg albumin. If, however, the results are considered due to the effect recently reported (15)

TABLE III

Observations on Dissolved Egg Albumin-Anti-Egg Albumin Precipitates

Experiment No.	Material used	Analytical concentrations		Concentrations from sedimentation diagrams		Mean $s_{20} \cdot 10^{13}$ principal globulin component	$s_{20} \cdot 10^{13}$ globulin component next in amount	Dispersity
		Total N	Antibody N	Ea	Ea-antibody*			
		mg. per ml.	*mg. per ml.*	*gm. per 100 ml.*	*gm. per 100 ml.*			
9	Ea-Anti-Ea precipitate dissolved in Ea	1.53	0.93	*ca.* 0.3 to 0.4	0.4	12.8	26.4	Polydisperse
10	Same, large excess Ea	1.82	0.65	0.54	0.6	9.0	7.2, 16.2, 19.1	"
11	First half precipitate, large excess Ea	3.4	1.4	0.73	1.00	9.7	12.3	"
12	Second half precipitate, less Ea	1.9	1.2	0.31	0.75	12.1	13.3	"
13	Solution 11, 3 days later	3.4	1.4	(0.7)†	1.25	9.9		"
14	First half precipitate, excess Ea	1.49		0.29	0.5	9.6		"
15	Second half precipitate, slight excess Ea	1.29		0.11	0.5	14.1	18.1, 10.7, 11.6	Several apparently homogeneous peaks

* Total concentration of all components with s above $7 \cdot 10^{-13}$.

† Uncertain.

they seem intelligible. Thus in Experiment 15 (Fig. 2 *e*), with only a very small excess of egg albumin, there are a number of almost homogeneous complexes in the solution, consisting apparently of relatively simple combinations between the antibody and the antigen. With increasing egg albumin concentration, as in the other experiments, these complexes would be in part dissociated, forming

increasing numbers of smaller molecules as the egg albumin concentration increases. The mean sedimentation constant would therefore decrease at the same time, as found experimentally. Since all sedimentation constants found, however, were larger than that for antibody alone, the results are consistent with the theory that soluble chemical compounds of antigen and antibody are actually formed. These findings are also consistent with the theory that specific precipitates are built up by the interaction of multivalent antigen (or hapten) with multivalent antibody to form large aggregates (2*d*, 2*e*; *cf.* also 16, 17), since the molecular species found after the precipitate has been dissolved with a minimum antigen excess are larger than those in solutions containing much antigen. The Svedberg ultracentrifuge has thus furnished new insight into the complex chemical reactions involved in the inhibition zone of the precipitin reaction. The extension of its use to other immune systems in which precipitation is not involved would appear promising.

Finally the present studies, by making available approximate molecular weights for certain antibodies, have made it possible to calculate actual molecular formulas for some of the compounds formed in specific precipitation. This phase of the work will be taken up in a separate communication.

We wish again to express gratitude to Professor The Svedberg for his kind extension of the hospitality of his Institute and for his freely given counsel, and to Dr. Arne Tiselius and the other members of Professor Svedberg's staff for their assistance and for many courtesies.

SUMMARY

1. Highly purified rabbit Type III pneumococcus anticarbohydrate proved to be homogeneous in the ultracentrifuge and its sedimentation constant, $7.0 \cdot 10^{-13}$, did not differ from that of the principal component of normal rabbit globulin or of immune rabbit globulin containing up to 50 per cent of anti-egg albumin. The molecular weight of antibody in the rabbit is therefore probably very close to that of the principal normal globulin component, namely, 150,000.

2. Highly purified horse Type I pneumococcus anticarbohydrate, on the other hand, was only homogeneous in the ultracentrifuge when prepared from sera stored without preservative. Its sedimen-

tation constant, $18.4 \cdot 10^{-13}$, coincided with that of the principal globulin component in most of the Felton solutions and purified antibody solutions studied. The molecular weight of pneumococcus anticarbohydrate in the horse is probably three to four times that of the principal normal globulin component.

3. The significance of the differences between pneumococcus anticarbohydrate formed in the rabbit and in the horse is discussed.

4. Results are given of ultracentrifuge studies on the molecular species in solutions of egg albumin-anti-egg albumin specific precipitates dissolved in excess egg albumin. The implications of the results are discussed.

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